

How to make haploid *Arabidopsis thaliana*.

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The haploid inducer.

The haploid inducer is a *cenh3-1* null mutant in the Col-0 ecotype that is complemented by a *CENH3* transgene called *GFP-tailswap* (*cenh3-1* is embryo lethal). It has a vegetative phenotype that varies in intensity depending on growth conditions. Rosette leaves are slightly curled, and it also has shorter internodes after bolting. The mutant is mostly male sterile, but has reasonable female fertility (about 60-70% of wild type in our hands). It can be maintained as a homozygote because it yields a few hundred seed per plant if treated carefully. Later siliques have higher fertility. Importantly, a majority (90%) of the offspring are diploids similar to the parent plant. We do observe a low frequency of aneuploid offspring which can be easily distinguished from diploid siblings by their distinctive phenotypes. We generally send seed collected from *cenh3-1/+ GFP-tailswap/GFP-tailswap* plants, so 1/4 will be the desired *cenh3-1 GFP-tailswap*.

Genotyping the *cenh3-1* mutation and the *GFP-tailswap* transgene.

cenh3-1 is a point mutation in the *CENH3* gene (also known as *HTR12*). The mutation is G161A relative to ATG = +1. We genotype it with the following dCAPS primers:

Primer 1: GGTGCGATTTCTCCAGCAGTAAAAATC

Primer 2: CTGAGAAGATGAAGCACCGGCGATAT

Total Amplicon length: 215bp

dCAPs restriction polymorphism with EcoRV

cenh3-1 mutant allele : Does not cut (215bp)

WT *CENH3* allele: digestion gives 191 bp and 24bp fragment

The polymorphism can be resolved by running the digested PCR products in a 2.5% gel

GFP-tailswap is a Hyg marked transgene on chromosome 1 (identified by TAIL PCR) that complements the embryo-lethal phenotype of *cenh3-1*. It has

the native *CENH3* promoter and terminator

a N-terminal GFP tag

the N-terminal tail domain of histone H3.3

the C-terminal histone fold domain of *CENH3*

We genotype *cenh3-tailswap* with the following primers.

Primer 1 for wild type and T-DNA : CACATACTCGCTACTGGTCAGAGAATC

Primer 2 for wild type only: CTGAAGCTGAACCTTCGTCTCG

Primer 3 for the T-DNA: AATCCAGATCCCCCGAATTA

WT (Primer 2 + Primer 1): 518 bp

TDNA (Primer 3 + Primer 1) = ~450bp.

Crossing the haploid inducer.

Haploid *Arabidopsis* are produced by crossing the haploid inducer to wild type. In the fertilized zygote, chromosomes from the mutant parent are eliminated at a high frequency, resulting in a substantial fraction of haploid plants in the F1 that carry only chromosomes from the wild type parent. The haploid inducer can be used as a male or a female parent in a cross. If used as a female parent in a cross, then entire female genome is eliminated in the resultant zygote and the haploids are purely paternal in origin. On the other hand, if it is used a male parent, the paternal genome is eliminated following fertilization and thus the haploids are maternal in origin.

Although it is possible to generate either maternal or paternal haploids, the frequency of haploids in the F1 is higher when the haploid inducer plant is used as the female parent. As the mutant is mostly male sterile, we don't usually emasculate before pollination. There will be a lot of aborted seed (~80%) if the haploid inducer is fertilized by wild types, so you will find many dark brown, "empty" and misshapen seeds.

Note that the procedure above will yield haploid plants in which the cytoplasm is derived from Col-0. If this is a problem, you can first cross a plant with desired cytoplasm as the female to the haploid inducer as the male, choose a fertile diploid hybrid F1 plant, then segregate a sterile diploid plant in the F2. Now you have a haploid inducer with the cytoplasm of your desired ecotype.

If crossing the haploid inducer as the male, we have found that some flowers have reasonable amounts of viable pollen, while others have virtually none. It helps to use a dissecting scope for the crosses, and to turn up the magnification to the point where you can see pollen being deposited on the stigma. If you use >10 mutant flowers per wild type stigma you can get 20-50 seeds per cross.

Selecting haploids in the F1 of a cross to the haploid inducer.

Among viable plants in the F1 you will find four types of plants, diploid hybrids, aneuploid hybrids (with >10 chromosomes), self fertilized plants (rare, these can occur if emasculation is not done) and haploids. When the haploid inducer is the male in a cross, we observe mostly diploid hybrids, ~5% haploids and a very low frequency of aneuploids. On the other hand, if the haploid inducer is used a female parent in a cross, we obtain 25-50% haploids, 25-50% aneuploids and 25% diploid hybrids.

We recommend planting the seeds on MS plates to maximize germination frequency. Even some of the misshapen ones may yield viable offspring, so plate everything. In our experience, late germinating seeds can be more likely to be haploid, so transfer everything that germinates to soil.

Haploid *Arabidopsis* are readily distinguished irrespective of ecotype. Rosette leaves are somewhat smaller than diploid, especially early in development. Leaves are also narrower than diploids. Haploids are vigorous, and these differences may diminish later on. The most obvious phenotypes are seen after bolting: flowers are very small (following the general pattern tetraploid > triploid > diploid > haploid), and the plants are sterile.

Aneuploids in *Arabidopsis* generally have distinctive developmental phenotypes that are more severe than haploids. In a large population you will see very similar phenotypes that represent the 5 trisomics - a pale green phenotype, a brassinosteroid-like phenotype, a very sick dark green phenotype e.t.c. Haploids are more like diploids than all of the aneuploids in terms of vegetative phenotype.

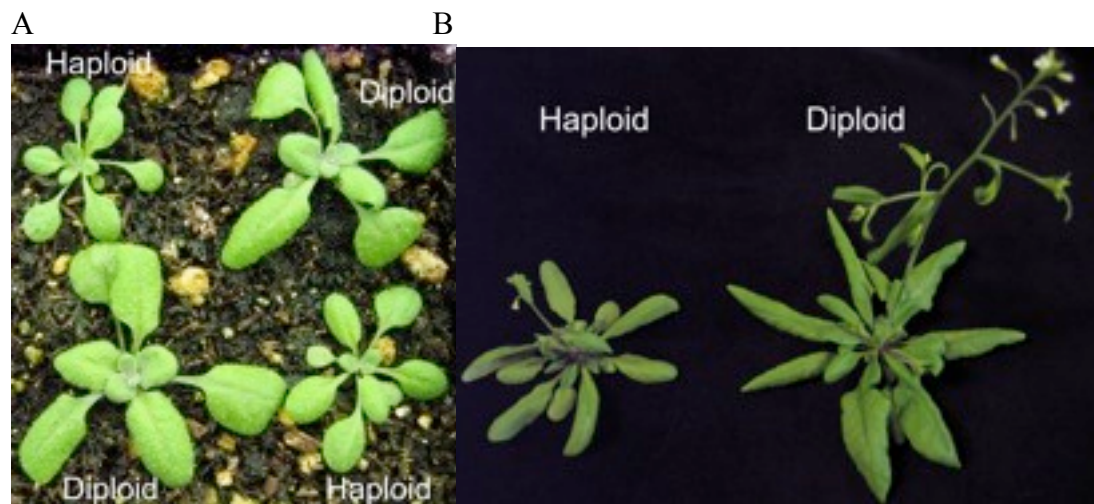


Figure 1A. Comparison of haploid and diploid plants 15 days post germination. 1B. Haploid and diploid plants after bolting. (All plants Col-0 ecotype).

In the absence of a phenotypic marker (see below), the easiest way to pick out the desired haploids is to wait until bolting and pick the sterile individuals. Diploid and aneuploid hybrids are fertile.

We have developed a haploid inducer carrying a dominant phenotypic marker, a seed expressed GFP (*At2S3 promoter:GFP*) created by Francois Parcy. Seed GFP from this transgene is very bright, and is easily scored with a fluorescent dissecting microscope. This allows you to discard fluorescent seeds from diploid and aneuploid hybrids. Non-fluorescent seeds will be haploids that lack the mutant-derived chromosomes. The *cenh3-1 GFP-tailswap pAt2S3:GFP* line is available on request.

Two explanatory notes:

- Centromere fluorescence from the GFP-tailswap protein is too weak to be seen through the seed coat, although it can be scored in root cells after germination.
- Monosomics that have lost just one chromosome from the haploid inducer are probably inviable (we have never observed them). These will not result in non-fluorescent seeds that give rise to viable plants.

We are also making a haploid inducer with a different dominant phenotypic marker, a transgene that causes chlorosis/white colour in vascular tissue (*SUC2* promoter driving *PDS* miRNA, made by John Bowman). This should be available sometime in Spring 2010.

Doubling haploids to create diploids.

To propagate plants with the desired haploid genotype, you will want to convert haploids to diploids. You can treat haploid plants with colchicine before or after bolting (before is better). Simply make a solution of 0.25% colchicine, 0.2% Silwet and drop 20 μ L on the meristem. The meristem will appear to die, and the plant may become very sick. After it recovers, you will find fertile inflorescences that yield diploid seeds.

An even easier way is to collect spontaneous diploids from untreated haploid plants. Haploids have 5 unpaired chromosomes, and occasionally all 5 will segregate to the same side of the spindle during meiosis I, giving viable gametophytes. As haploids are sterile, they will tend to flower indeterminately if left to grow, increasing the chances of obtaining spontaneous doubled haploids by meiotic non-reduction. We routinely collect >50-500 diploid seeds from each haploid plant.

As haploids produce very little pollen, it is easy for them to outcross to other plants in the room. To avoid this, we recommend growing haploids apart from fertile diploid *Arabidopsis thaliana*. The best approach is probably to use *At2S3:GFP* selection and only plant the haploid seeds, in a separate growth chamber dedicated to haploid plants. If you are selecting haploids from a mixed population of viable plants, you could kill the diploids and fertile aneuploids as soon as you see silique elongation. One could also take steps to minimize outcrossing (putting haploid plants in aerocons or even better using a bagging system).

References

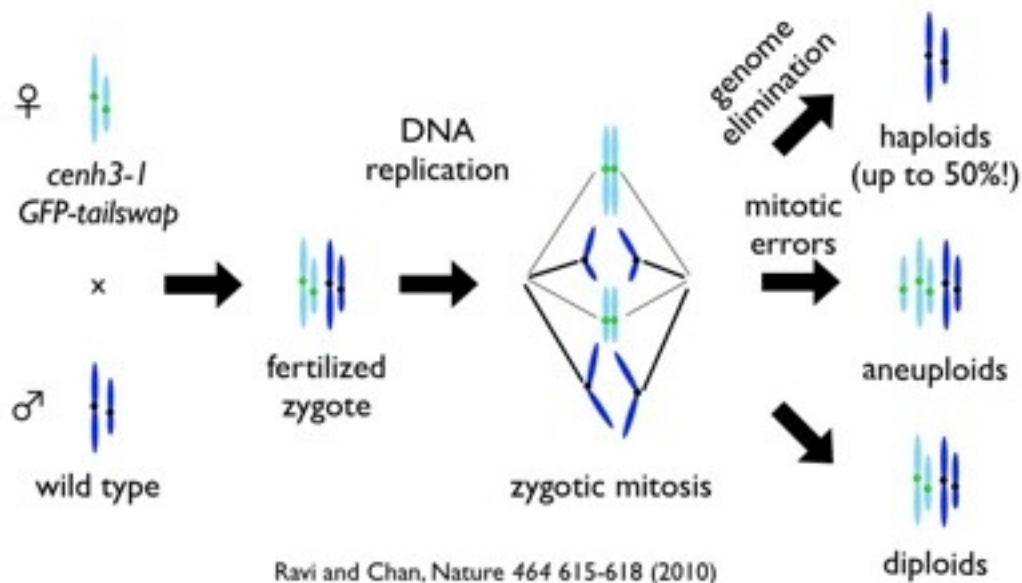
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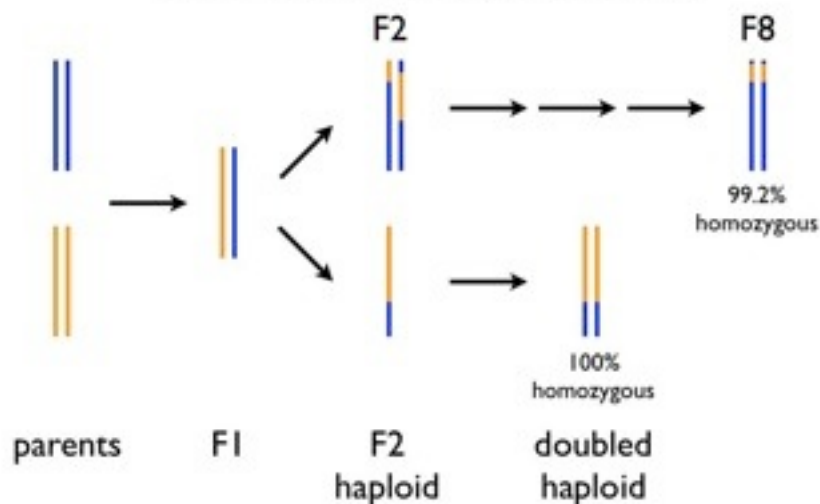
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 Genetics, 186, 461–471 (2010)

Haploid plants produced by crossing *cenh3* mutants to wild type



Haploids in plant breeding: instant homozygous lines



I) Haploids for exploring natural variation

- ✿ Cross haploid inducer to desired F1 (or F2, F3 e.t.c.).
Create homozygous recombinant lines without inbreeding.
- ✿ Reproducible: at least 8 different labs have made haploids from 6 strains
- ✿ Make QTL mapping populations quickly from the **most interesting** accessions

2) The fastest way to make multiple mutants

a/A b/B c/C d/D e/E f/F g/G h/H 8 heterozygous unlinked loci

↓ cross to *GFP-tailswap*

a b c d e f g h

↓ collect diploid progeny,
convert haploid to diploid

a/a b/b c/c d/d e/e f/f g/g h/h 1/256 doubled haploids

vs.

1/65536 segregating diploids

3) Adult plants from female-gametophyte lethal mutants

♀

♂

GFP-tailswap × *m / M*

↓

Identify haploids. Predict:

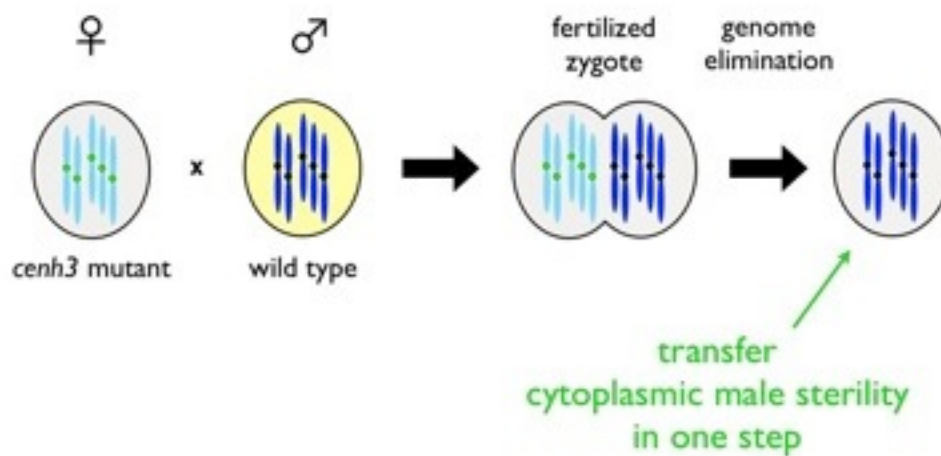
50% *m* haploids (are they alive?)

50% *M* haploids

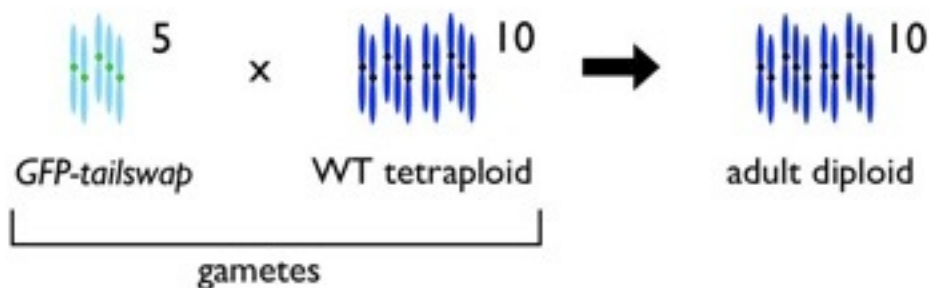
✿ *m* is female gametophyte lethal, can't be homozygous diploid.

✿ in principle, make haploid adults from male gametophyte-lethal mutants

4) Transfer any nuclear genome into any cytoplasm



5) Convert tetraploid *Arabidopsis* to diploid (and haploid)



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all
Wa-I
accession